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(54) Title: METHOD AND MEDIA FOR ENHANCING VIABILITY, MATURATION, AND CRYOPRESERVATION OF CELLS

(57) Abstract

A method to increase viability, number, survival and maturation of cells for transplantation or cryopreservation by culturing the cells with Sertoli cells or with Sertoli-cell conditioned media (SCM) prior to transplantation (pre-culturing) or cryopreservation.

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METHOD AND MEDIA FOR ENHANCING VIABILITY, MATURATION, AND CRYOPRESERVATION OF CELLS

BACKGROUND OF THE INVENTION

TECHNICAL FIELD

The present invention generally relates to cell transplantation and specifically to methods of improving cell viability, graft survival, the viability of cryopreserved cells and providing increased numbers of differentiated cells for transplant.

BACKGROUND ART

Transplantation of cells and tissues is being utilized therapeutically in a wide range of disorders from cystic fibrosis (lungs), kidney failure, degenerative heart diseases to neurodegenerative disorders. Improved means to facilitate such transplants are needed, particularly where differentiated cells are being transplanted. Generally these cells cannot be cultured to increase cell number so preservation of viability for transplant is critical. Further, the number of differentiated cells available for transplant is often low and methods of increasing the number and/or availability of such cells are also needed.

Transplantation protocols in addition to transplanting tissues and/or organs can include the infusion of cell suspensions from a donor. A wide range of transplantable material either currently being transplanted or contemplated for transplants includes skin grafts, corneas, hepatic tissue, kidneys, hearts, islet cells, neurons, bone, bone marrow, and the like. The obligatory step for the success of this kind of treatment is to have enough viable cells or organs available for the transplant.

For example, in some cancer therapies a patient's bone marrow is removed, and then reinfused following high dose chemo- and/or radiation therapies. It would be useful to have improved methods of preserving such autologous cells. In other cases, bone marrow must be from donors. Often there is not a match from relatives and the marrow must be matched from the national registry. It would be useful to be able to have preserved such cells rather than having to find the donor at the time the cells are needed. Therefore, improved methods of cryopreservation are needed since a substantial portion of cryopreserved cells are not viable upon thawing.

In addition, hybridomas are stored utilizing cryopreservation. Improved methods for preserving hybridomas with increased viability would be useful.

As a further example, the central nervous system (CNS) (brain and spinal cord) has poor regenerative capacity which is exemplified in a number of neurodegenerative disorders. An example of such a disorder is Parkinson's disease. The preferred pharmacotherapy for Parkinson's disease is L-dopa which helps the symptoms of this disease in humans. However, the neuropathological damage and the debilitating progression is not reversed by this pharmaceutical treatment protocol.

Laboratory and clinical studies have shown the transplantation of cells into the CNS is a potentially significant alternative therapeutic modality for neurodegenerative disorders such as Parkinson's disease (Wictorin et al., 1990; Lindvall et al., 1990; Sanberg et al., 1994; Bjorklund and Stenevi, 1985; Freeman et al., 1994). In some cases, transplanted neural tissue can survive and form connections with the CNS of the recipient, i.e. the host (Wictorin et al., 1990). When successfully accepted by the host, the transplanted cells and/or tissue (i.e. the graft) have been shown to ameliorate the behavioral deficits associated with the disorder (Sanberg et al., 1994). The obligatory step for the success of this kind of treatment is to have enough viable cells available for the transplant.

Currently, fetal neural tissue is the primary graft source for neural transplantation (Lindvall et al.,

1990; Bjorklund, 1992; Isacson et al., 1986; Sanberg et al., 1994). Other viable graft sources include adrenal chromaffin cells and various cell types that secrete neural growth factors and trophic factors. The field of neural tissue transplantation as a productive treatment protocol for neurodegenerative disorders has received much attention resulting in its progression to clinical trials. Preliminary results and clinical observations are promising but obtaining enough viable cells remains a problem.

For example, one treatment for Parkinson's disease (PD) intracerebral transplantation therapy, has accentuated research interest in restoring some of the circuits in the nigrostriatal pathway [Lindvall et al., 1990; Lindvall, 1994; Freeman et al., 1995; Kordower et al., 1995, 1996]. While the initial findings are encouraging and have resulted in behavioral improvements in patients with PD, the current clinical protocols for intracerebral transplantation have to be improved in terms of increasing short- and long-term survival of embryonic dopaminergic (DA) cells, and to find alternative graft sources to avoid the problem with lack of donor tissue obtained from elective abortions.

Lately, research has focused on finding trophic factors, able to increase the survival of DA cells prepared for transplantation, maintain the *in situ* survival post-transplantation of embryonic DA neurons

transplanted into the striatum, as well as increase graft volume, and thereby re-innervate a larger part of the caudate and putamen which has been shown to have effect both *in vitro* and *in vivo*. Trophic factors such as NGF, bFGF, EGF, IGF I and II, TGFβ1-3, PDGF, BDNF, and GDNF [Engele and Bohn, 1996; Mayer et al., 1993; Knusel et al., 1990, 1991; Poulsen et al., 1996; Nikkhah et al., 1993; Othberg et al., 1995; Hyman et al., 1991, 1993; Lin et al., 1993] have been investigated and shown to have pronounced effects *in vitro*, however the effects *in vivo* have yet to be further established. Reversal of MPTP and 6-OHDA lesions in primates [Gash et al., 1996] and rats, as well as increased graft survival, have been demonstrated by the addition of NGF or bFGF to the cell suspension prior to grafting [Chen et al., 1996; Dunnett et al., 1995], or by transplanting neurons derived from a neural progenitor (CINP) cell line, transduced retrovirally with NGF [Martinez-Serrano et al., 1995] and astrocytes transduced with BDNF [Yoshimoto et al., 1996]. GDNF has been shown to increase graft survival, extend fiber outgrowth and alleviate behavioral effects after 6-hydroxydopamine lesions in the striatum of rats [Sauer et al., 1994; Johansson et al., 1995; Bowenkamp et al., 1995; Rosenblad et al., 1996; Olson, 1996].

In treating disease it is often useful to treat tissue locally, rather than systemically, with trophic factors, particularly areas of tissue damage as for

example in wound healing. Additionally, it is becoming increasingly recognized that multiple trophic factors acting in concert are likely to be necessary for successful treatment. Further, the availability of multiple trophic factors at various time points during treatment may be necessary to enhance successful treatment.

Long term maintenance of functional recovery has been observed in a diabetic animal model utilizing a novel transplantation treatment protocol utilizing isolated islet cells and Sertoli cells. It is clear that the efficacy of the treatment is due to the presence of the Sertoli cells, in part, due to their known immunosuppressive secretory factor [Selawry and Cameron, 1993; Cameron et al., 1990]. However, Sertoli cells are also known to secrete a number of important trophic growth factors.

Accordingly, it would be desirable to utilize Sertoli cells as a source for trophic factors to improve viability and growth of cells/tissues for transplantation, cryopreservation and for trophic factor support of damaged tissue. Sertoli cells actively participate in the genesis of spermatozoa. The Sertoli cells make a wide variety of nutritive, trophic and regulatory proteins, amongst them a wide variety of trophic factors and their receptors [Skinner, 1993]. Individual trophic factors, as listed in Table 1, have

been evaluated, but biological requirements are complex and the interaction of various components often necessary to effectively provide the necessary stimulants. It would be useful to design preparations that provide the various components and interactions to effectively improve viability, maturation, number and growth of cells/tissues for transplantation and for wound healing and cryopreservation.

Cell transplantation therapies are optimized by the availability of cryopreserved cells which have high viability. Transplantable cells, such as fetal brain cells, do not withstand cryopreservation well. Therefore, it would be desirable to have a method for enhancing the preservation and viability of cryopreserved cells in order to optimize the function of the cells and to obtain the resultant benefits to the transplant recipient.

SUMMARY OF THE INVENTION AND ADVANTAGES

In accordance with the present invention, there is provided a method of enhancing the viability of cryopreserved cells including the steps of culturing Sertoli cells in media to produce Sertoli-cell conditioned media (SCM), adding the conditioned media to cells to be cryopreserved and then cryopreserving the cells.

In a further embodiment, the method includes the steps of co-culturing Sertoli cells and cells to be cryopreserved and then cryopreserving the cells.

Also in accordance with the present invention, there is provided a medium for enhancing the viability, maturation and cryopreservation of cells. The medium is generated by the steps of culturing Sertoli cells in media to produce Sertoli cell conditioned medium (SCM) and removing the SCM from the Sertoli cells.

The present invention also provides a method to increase survival and maturation of cells for transplantation by the step of culturing the cells with Sertoli cells or with sertoli-cell conditioned media.

Also in accordance with the present invention a method of improving survival of a graft *in situ* by treating the graft *in situ* with sertoli-cell conditioned media or Sertoli cells is provided. Also the method of the present invention can be use to improve wound healing *in situ* by treating the wound *in situ* with sertoli-cell conditioned media.

The method of the present invention provides a means to induce phenotypic change in cells for transplantation by culturing the cells to be transplanted with sertoli cells or conditioned sertoli cell media.

BRIEF DESCRIPTION OF THE DRAWINGS

Other advantages of the present invention will be readily appreciated as the same becomes better understood by reference to the following detailed description when considered in connection with the accompanying drawings wherein:

FIGURE 1A-E are bar graphs illustrating the viability of post thaw cryopreserved (**A**) rat fetal brain cells (FBC) cryopreserved with rat Sertoli cells, (**B**) rat fetal brain cells (FBC) cryopreserved with porcine Sertoli cells, (**C**) hNT cells cyropreserved with porcine Sertoli cells and (**D**) the effect on the number of cells, (**E**) increased survival of post-thaw co-cryopreserved E15 Tyrosine hydroxylase positive dopaminergic neurons and rat Sertoli cells, the number of live vs. dead cell estimates were made one hour following thawing, * denotes $p<0.05$ with Students t-test;

FIGURES 2A-C are light interference micrographs illustrating cells from the ventral mesencephalon of fetal rats (VM) isolated and cultured for seven days in control medium (CM) or Sertoli cell pre-conditioned medium (SCM) and photographed with darkfield, interference contrast optics, wherein (**A**) depicts VM cells incubated in CM showing no evidence of stimulation or differentiation, (**B**) depicts VM cells incubated in SCM appearing highly stimulated, and (**C**) at higher

magnification, depicts VM cells incubated in SCM exhibiting neurite outgrowth;

FIGURES 3**A-B** are photomicrographs of tyrosine-hydroxylase (TH) immunostained cultures in (**A**) control cultures and (**B**) rat Sertoli-cell conditioned media treated cultures at 7 days *in vitro*, wherein arrows indicate TH-positive cells, magnification ×100;

FIGURE 4 is a bar graph showing the effect of rat Sertoli conditioned media on the yield of tyrosine hydroxylase (TH)-positive neurons as 7 days *in vitro* wherein data represents the mean ± S.E.M. of four independent culture experiments, * denotes, $p<0.028$, significant difference from control;

FIGURES 5**A-B** are photomicrographs of tyrosine-hydroxylase (TH) immunostained (**A**) E15 ventral mesencephalic cultures alone and (**B**) porcine Sertoli-cell co-cultures at 7 days *in vitro*, arrows indicate TH-positive cells, magnification ×200;

FIGURE 6**A-D** are bar graphs showing the effect of co-culture with porcine Sertoli cells on the yield of rat (E15) fetal mesencephalic TH-positive neurons at (**A**) 7 day *in vitro*, (**B**) size of soma area, (**C**) length of the longest primary neurite, and (**D**) number of primary neurites per cell wherein data represents the mean ± S.E.M. of four independent culture experiments, * denotes, $p<0.0001$, significant difference from control;

FIGURE 7 is a bar graph showing the effects of porcine Sertoli and Peritubular cells on rat E15 ventral mesencephalic cultures;

FIGURE 8 is a bar graph showing the effect of previously cryopreserved Sertoli cells on rat E15 ventral mesencephalic DA neurons;

FIGURE 9 is a bar graph showing the effect of porcine Sertoli cells co-culture on absolute number of DA neurons from hNT neuron culture; and

FIGURE 10A-B are photomicrographs of tyrosine hydroxylase (TH)-positive hNT neurons in (**A**) control and in co-culture with (**B**) porcine Sertoli cells isolated from 2.5 month old animals, note extended neurite outgrowth in (**B**), arrow TH-positive neuron, magnification x100.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

The present invention provides a method to increase viability, number, survival and maturation of cells for transplantation or cryopreservation by culturing the cells with Sertoli cells or with sertoli-cell conditioned media (SCM) prior to transplantation (pre-culturing) or cryopreservation.

By use of the term cells it can include tissue including organs as well as suspensions of separated cells as will be apparent from the text. The cells or

tissue upon transplantation can be referred to as a graft. The cells for transplantation can include but are not limited to islets cells for diabetes; myoblasts for muscular dystrophy; human or animal neurons for stroke, brain and spinal cord injury, Alzheimer's disease, Huntington's disease and other neurodegenerative disorders; septal and GABAergic cells for epilepsy; chromaffin, ventral mesencephalic or other dopaminergic cells for treatment of Parkinson's Disease; trophic factor secreting cells for neurological disorders; hepatocytes for liver disease; skin grafts for wound healing and/or burns, and bone marrow or stem cells for hematopoietic and genetic disorders.

The method of the present invention can be utilized with transplantable cells from tissues such as endocrine cells, muscle cells, and other cells by utilizing similar techniques as those described herein for neural cells. Furthermore, the method of the present invention may be used for enhancing the outcomes of cell transplant, such as myoblast transplants and cells for gene therapy, by providing such cells with enhanced viability and tropic support for transplant. That is, Sertoli cells and the trophic factors which they produce and/or secrete into the conditioned media are used to facilitate cell viability and maturation and transplant survival and graft function of the cells being transplanted.

Further, the present invention provides in an embodiment that the cells to be transplanted are dopaminergic neuronal cells which are generally of fetal or embryonic origin as discussed herein above for Parkinson's disease. In a further embodiment the absolute number of dopaminergic neural cells in culture is increased utilizing the method of the present invention from a human teratocarcinoma cell line as shown in the Examples.

Maturation of the cells to be transplanted includes differentiation of the cells and can include changes in density of cell surface receptors as well as induction, distribution and quality of cell surface receptors and other markers of cell activity. This can be referred to as a change in phenotype of the cell. These changes will be dependent on the type of cell being cultured and can include changes in phenotype in addition to cell surface receptors. For example, for neural cells the phenotypic changes can include prolongation of axons and neurite outgrowth, changes in pattern of neurite branching, changes in number of growth cones and processes, changes in synaptic transmitter substances and nuclear shape as shown in the Examples.

As discussed herein, there is a need for differentiated viable cells for transplantation. The method of the present invention, by culturing with sertoli cell conditioned media or with sertoli cells

allows for the maturation, i.e.change in phenotype, of the cells so that adequate numbers of differentiated cells are available for transplantation.

The present invention also provides methods for enhancing the viability of cryopreserved cells by culturing Sertoli cells or Sertoli-cell conditioned medium with the cells to be cryopreserved. Alternatively, the method of the present invention provides for adding the conditioned medium to cryopreserved cells upon thawing.

Enhanced viability describes the ability of cells to survive and function normally following a sustained period of cryopreservation. This can also be referred to as post-thaw viability. That is, cells which are harvested and cryopreserved for later use for purposes including transplantation, can be thawed and a greater percentage of the cells will be alive and viable retaining their original functions. For example, differentiated, non-dividing cells can be harvested for transplantation, cryopreserved according to the present invention, and thawed resulting in a substantially greater percentage of live cells than by previous methods of cryopreservation.

By cryopreserved or cryopreservation, it is meant that the cells are stored at temperatures which are low enough to prevent normal biological functions from occurring. Generally this temperature is at least -70°C

and lower under liquid nitrogen. At these low temperatures, biological degradation of the cells is inhibited thereby preserving the functions of the cells. However, as described above, the prior art methods of cryopreserving cells result in a substantial proportion of cells failing to survive or be viable following cryopreservation. Typically, cryopreservation involves storing cells in a medium which may contain DMSO or an equivalent at very low temperatures by refrigeration or storage under liquid nitrogen or other types of cooling, as known to those skilled in the art.

The cells which may be cryopreserved with Sertoli cells or in SCM, according to the present invention, include but are not limited to cells of the peripheral and central nervous system including neural cells, lymphocytes, hybridomas, fibroblasts, cells for gene therapy, fetal cells from various tissues, myoblasts, hepatocytes, endocrine cells, endothelial cells and the like.

As shown in the Examples herein below, the cells preserved or produced in accordance with the present invention can be transplanted into the CNS (brain) to replace dysfunctional cells and when co-transplanted with Sertoli cells, can avoid being rejected. Such a protocol results, therefore, in increased cell survival and cell functional integration with the host tissue. This then will promote re-

establishment of normal neural tissue function and thereby ameliorate the behavioral and functional deficits associated with the neurological and/or neurodegenerative disorder being treated.

The present invention also provides a method of producing a sertoli-cell conditioned medium (SCM). The source of Sertoli cells is by primary cell isolation from the mammalian testis. The protocol for harvesting the cells is as set forth by Cameron and Muffle (1991) and by Griswold (1992). The method of the present invention can be used with Sertoli cells from any suitable mammalian source such as rat or porcine. However, if available and suitable, human Sertoli cells may be utilized.

The conditioned medium is prepared by culturing Sertoli cells in a suitable culture medium (incubation medium) as described herein. Isolated Sertoli cells are cultured in incubation medium from one hour to seven days and at a cell density of 1×10^4 to 1×10^7 cells/cm^2 at 39°C with 5%CO_2-95% air. In a preferred embodiment the isolated Sertoli cells are cultured in incubation media for 48 hours at a density of 6×10^5/cm^2 and at 39°C with 5%CO_2-95% air. It should be noted that time versus cell density as well as culture temperature can be adjusted in determining the culture conditions can be adjusted as known to those skilled in the art such that the conditioned media is standardized between species as needed.

Following incubation of the Sertoli cells in the incubation medium, the medium (SCM) is deemed "conditioned". That is, following the conditioning of the medium, the medium contains nutritional, immunosuppressive, and other factors from Sertoli cells defined herein as trophic factors which enhance not only the viability of cells, but which imparts enhanced growth characteristics and can induce changes in phenotype to the cells cultured in the conditioned medium. The Sertoli cells are generally removed from the conditioned media of the present invention following the culturing of the Sertoli cells therein.

For cryopreservation, the Sertoli cells can be removed from the conditioned media of the present invention following the culturing of the Sertoli cells therein, or, can be left in the conditioned media to further enhance the cells to be cryopreserved. Additionally, the cells to be cryopreserved can be co-cultured with the Sertoli cells prior to cryopreservation. Furthermore, the conditioned medium containing the trophic factors can be added to other media for cell culturing and cryopreservation.

In general the incubation media used to culture the sertoli cells is as set forth in the Examples herein below and after an initial culture with serum-containing media (as indicated in the text) the incubation media during the final culture is a serum-free media. However,

where appropriate for human use or veterinary use the initial culture may be with species specific serum. In an embodiment the serum-free media is either X vivo-10 or X vivo-15 media (Whittaker Bioproducts). This is a serum-free and FDA-approved media for IL-2/LAK infusions in patients.

Following incubation the conditioned media is collected. Matched species serum albumin can be added to stabilize the conditioned media if necessary. The conditioned media is stored at 4°C to -70°C, depending on when it will be used.

The present invention provides for a conditioned media in a preferred embodiment produced by culturing sertoli cells in a serum-free culture media for 48 hours and at a cell concentration of 6×10^5 cells/cm^2 or alternatively in a combination of time and cell density providing the same conditioned media. The conditioned media is standardized by bioassay. In general cells of interest are cryopreserved and/or observed for changes in phenotype in each lot of the conditioned media. The conditioned media must provide the same post-thaw viability, cell number increase or phenotypic changes as have been determined for the initial use of the conditioned media.

The conditioned media is further characterized by measuring the trophic factor content by bioassay and appropriate immunoassays or other assays. Sterility is

tested by culture in thioglycolate broth and endotoxin measured by limulus lysate assay as is known in the art. Where necessary DNA and virus exclusion, if needed, will employ such techniques as ultrafiltration, column chromatography, virasol, ethanol fractionation, polyethylene glycol/bentonite precipitation, gamma irradiation, and/or solvent/detergent treatment as has been used for intravenous gamma globulin and monoclonal antibodies. Each lot of conditioned media is standardized either by concentration or amount administered so that comparisons can be made.

The present invention also provides a method of improving survival of a graft *in situ* by treating *in situ* with sertoli-cell conditioned media, generally at the time of transplant and can also include the use of Sertoli cells as appropriate. The method also encompasses improving wound healing *in situ* by treating *in situ* with sertoli-cell conditioned media either topically or by site-directed injection. In an embodiment the wound to be treated is an injured spinal cord, brain or skin wound.

The conditioned media utilized in the present invention, is administered in combination with other drugs or singly consistent with good medical practice. The composition is administered and dosed in accordance with good medical practice taking into account the clinical condition of the individual patient, the site

and method of administration, scheduling of administration, and other factors known to medical practitioners. The "effective amount" for purposes herein is thus determined by such considerations as are known in the art. The amount must be effective to achieve improvement including but not limited to prolonged graft survival or improved wound healing rate or length and other indicators as are selected as appropriate measures by those skilled in the art.

The present invention provides a method to induce phenotypic change in cells for transplantation by culturing the cells to be transplanted with sertoli cells. The cells are pre-cultured with the sertoli cells in a culture media for seven days and at a cell concentration of 1×10^5 cells/cm^2 for the cells to be transplanted and at a cell concentration of 2.5×10^5 cells/cm^2 for the sertoli cells. The cells to be transplanted are then harvested, isolated and transplanted. Adjustments in culture conditions as are known to those skilled in the art to accommodate species differences can be made.

In an embodiment as shown in the Examples, the method utilizes cells from a human teratocarcinoma neuronal cell line and using the method of the present invention the absolute number of tyrosine hydroxylase positive neurons in the culture is increased. This

method thereby increases the absolute number of dopaminergic neural cells available for transplant.

Utilizing fetal rat ventral mesencephalic neurons (VM) and human postmitotic neurons (hNT) derived from a cell line applicants demonstrate in the Examples herein below that culturing such cells with Sertoli cells or Sertoli cell conditioned media enhanced survival of fetal dopamine neurons and it increased neuritic outgrowth and cell body area as well as increasing post-thaw viability after cryopreservation.

Further, it was unexpected found in culturing the hNT cells that the absolute number of dopamine neurons (tyrosine-hydroxylase, TH, positive) increased. The culture conditions with the Sertoli cells or Sertoli cell conditioned media changed the phenotype of neurons in the culture to be dopamine neurons.

As shown in the Examples the survival of neurons is promoted by the stimulation of direct co-culture with Sertoli cells or sertoli cell conditioned media, which have a direct trophic effect on soma size, neurite outgrowth and can cause a change of phenotype *in vitro*. The average TH-positive cell number, and measurements of soma size, neurite outgrowth and number of branching points of the embryonic DA neurons treated with Sertoli cell co-culture, or culture in Sertoli cell conditioned media, were significantly increased after 7 days *in vitro*.

These data suggest that Sertoli cells can provide a constant secretion of trophic support, important for the survival and maturation of embryonic DA neurons *in vivo*. The change of phenotype seen in the human cell line, also provide additional support that certain proteins or gene products secreted by non-CNS Sertoli cells play an important role for development and maturation of the CNS.

Moreover, the phenotypic change as seen by the induced increased number of TH-positive neurons in the hNT-neuronal co-cultures were also seen with rat VM cells in Sertoli cell co-culture. The cells responded in co-culture in a ratio-dependent manner and the optimal ratios for VM cells were 1:5-1:1 (Sertoli:VM) and for hNT neurons, the ratio 5:1 (SC:hNT) was most potent.

A hypothesis for the above observations can be made, but it is not to be construed as limiting the present invention to this one mode of action. An early viability decline in the VM cell preparation may be a crucial factor for the poor survival of such transplants in the clinical intracerebral grafting protocols in Parkinson's disease (PD) treatment. It is therefore important to improve the *in vitro* cell viability in order to yield a higher number of surviving DA neurons which could expand the reinnervation of the striatum by increasing graft survival.

Continuing improvement has been seen only in PD patients receiving at least 3 to 4 embryonic VMs per side of the brain [Lindvall, 1994; Freeman et al., 1995]. The large number of embryonic donors required per patient is due to the fact that only 5-20% of the DA neurons survive [Sauer and Brundin, 1991; Kordower et al., 1996] the implantation. The cause of the poor survival may be due to the initial tissue dissection could produce axotomy of DA neurons, which might lead to retrograde cell death. Moreover, the long term underlying disease process in PD, may cause detrimental challenges on the transplanted DA neurons and an allograft immune response which could induce a rejection of the transplanted cells. Therefore, if this damage is reduced, it should increase survival of transplanted DA neurons.

Although many trophic factors have been reported to have an effect on DA neurons *in vitro*, there has only been a few successful reports about the *in vivo* effects of growth factors. The dilemma with long-term administration of trophic factors has been approached by either generating trophic factor secreting cells [Yoshimoto et al., 1995; Martinez-Serrano, 1996], or by adjunction of neurotrophins into different matrixes such as GDNF in fibrin glue [Johansson et al., 1995], and hence be able to provide neurotrophic secretion *in vivo*. However, there is still no evidence for effects of long-term administration, which will be preferable to be able

to support transplanted tissue in the brain. The fact that rat and porcine Sertoli cells have effect on survival and maturation of both rat VM neurons and hNT neurons, support Applicants earlier findings that it is possible to co-transplant neurons and Sertoli cells into the brain. The Sertoli cells can survive in the brain and provide for a life-time secretion of trophic factors and locally induced immunosuppressive factors.

The desert hedge hog (dhh) gene plays an important role in pattern formation of embryonic structures and has been shown to be present in developing and mature Sertoli cells [Bitgood et al., 1996]. It has also been shown that the hedgehog gene family has had a substantial effect on survival of DA neurons *in vitro* [Miao et al., 1996]. This suggests that Sertoli cells can induce an up-regulation of the dhh gene in the DA neurons and thus increase trophic factor secretion.

Prosaposin has been shown to be present in rats at birth and increase gradually in brain contents after postnatal day 10 when synaptogenesis begins to take place [Kotani et al., 1989]. It has been demonstrated that Sulfated glycoprotein-1 (SGP-1), homologous to Prosaposin in rat Sertoli cells, is released at specific stages of spermatogenesis [Collard et al., 1988], and have been proposed to be secreted by lysosomes [Igdoura et al., 1996]. Prosaposin has been detected in adult brain and testis by Northern blot analysis [Morales et al., 1996].

The trophic properties of Prosaposin has been demonstrated on murine and human neuroblastoma cells *in vitro* [O'Brien et al., 1994] showing neuritic outgrowth. Recently, studies of hippocampal CA1 neurons, have demonstrated that prosaposin has effect on rescuing those neurons after ischemic insult both *in vitro* and *in vivo* [Kotami et al., 1996].

Sertoli cell secreted trophic factors are potent for increased survival and morphological changes *in vitro*, and should, therefore provide an unique *in situ* long term delivery of trophic molecules when co-transplanted with embryonic DA cells or with other grafts.

The present invention provides in one embodiment for the co-culturing of Sertoli cells and a second cell type to be cryopreserved in media and cryopreserving the co-cultured cells together. Upon thawing, the Sertoli cells are therefore present and would be co-transplanted with the second cell type. Such a co-cellular transplant provides additional advantages.

Sertoli cells provide local immunosuppression by secreting an immunosuppressant agent, so that there would be no successful antibody or cellular immunological attack waged against the transplanted cells, including the Sertoli cells themselves. Additionally, since the immunosuppression is local and by a biologically tolerable agent, the side effects associated with both

systemic immunosuppression and cytotoxicity of agents such as CsA would be avoided. Hence, Sertoli cell co-transplantation provides a significant improvement over the use of systemic immunosuppression with CsA as the necessary adjunctive therapy to neural transplantation as shown in the example below.

The localized immunosuppression by a Sertoli cell-derived immunosuppressant agent can facilitate the survival of both cellular xenografts and allografts. With allografts, co-transplantation with Sertoli cells should provide localized immunosuppression so as to eliminate the need for systemic immunosuppression. With xenografts, co-transplantation with Sertoli cells can provide sufficient local immunosuppression so as to eliminate the need for systemic immunosuppression or the Sertoli cells may be used in combination with a systemic immunosuppressant at a lower dose to prevent rejection. When co-transplanted, the Sertoli cells not only provide local immunosuppression but provide trophic support (i.e. regulatory, nutritional and other factors) to the co-transplanted cells (i.e. the graft). Therefore, the Sertoli cells will not only provide inhibition of the immune response, but will allow enhanced growth and viability of allografts and xenografts by concomitant trophic support.

The above discussion provides a factual basis for the use of sertoli-cell conditioned medium and

Sertoli cells for preculturing cells to be transplanted prior to transplantation to increase survival and maturation and for cryopreservation. The methods used with and the utility of the present invention can be shown by the following examples.

EXAMPLES

GENERAL METHODS:

Rat embryonic ventral mesencephalic and porcine Sertoli cell co-cultures: Tissue to obtain ventral mesencephalon (VM) was collected from time pregnant rats, embryonic day 15 (E15; Civic Miller). The litter, after removal of the amnion, was placed into Hank's balanced salt solution (HBSS; Life Technologies) + 15mM Hepes (Life Technologies). The CNS was dissected out and VM was localized and divided along the midline and a cell suspension was prepared as described earlier [Othberg et al., 1995]. Briefly, the tissue was chemically dissociated in 0.1% trypsin (Sigma) and 0.05% deoxyribonuclease (Dnase; Sigma) in HBSS+15 mm Hepes 20 minutes at 37°C. The trypsin was removed and the tissue was rinsed 5 times in 0.05% DNase followed by a mechanical dissociation by the use of 1 ml automatic pipette into a single cell suspension. The cell number was estimated by a trypan blue dye exclusion method and was thereafter seeded at a concentration of 100,000 cells/cm^2, in poly-L-lysin (Sigma; 10μg/ml) coated 48 well

plates (Costar). The cells were plated in plating medium (DMEM:F12 supplemented with 3 mg/ml L-glutamine (Sigma), 0.01 ml/ml gentamicin sulfate (Gibco), 50 ng/ml retinol (Sigma), and 0.01 ml/ml insulin-transferrin-selenium (ITS, Collaborative Research, Inc.))

The dose/ratio response curve was performed by mixing porcine Sertoli cells:VM cells in the following ratios: 1:100, 1:10, 1:2.5, 1:1, 2.5:1, 5:1. The control for the dose-response was no Sertoli cells and 100,000 VM cells/cm^2 in medium as described. Applicants used either freshly isolated or cryopreserved porcine Sertoli cells in the dose response study.

<u>hNT neuron and culture</u>: The hNT cell line (Stratagene®, catalog number 204104) is derived from a human teratocarcinoma cell line (NT2), induced by retinoic acid to differentiate *in vitro* into postmitotic neurons (hNT) [Pleasure et al., 1993]. Briefly, the NT2 (NTera2/D1) cell line represents a committed neuronal precursor stage of differentiation. The NT2 cells are induced by retinoic acid (RA) to differentiate *in vitro* into postmitotic central nervous system neurons (hNT). In the course of the differentiation process, the NT2 cells lose neuroepithelial markers and gain markers specific for mature neurons. The hNT neurons are produced by culturing of NT2 cells in DMEM supplemented with glutamine and 10% (v/v) FBS. The cells are grown for five weeks in the presence of RA and then harvested by

mild trypsinization. The cells are replated and allowed to grow for several days wherein 10-20% have begun to differentiate into postmitotic neurons (hNT). Mitotic inhibitors are added to the medium to prevent overgrowth by undifferentiated cells and after ten day the hNT cells are harvested and used immediately or cryopreserved for later use.

During the process of maturation (differentiation) the NT2 cells loose neuroepithelial markers and gain those specific for mature neurons. After transplantation into athymic (nude) mice, hNT neurons can integrate and change phenotype into neurons similar to the target neurons and survive >14 months. [Kleppner et al., 1995]. These results suggests the usefulness of hNT neurons for screening the effects of trophic factors, similar to those lacking in the target area, thus providing evidence that Sertoli cells can provide such support.

Cryopreserved hNT neurons were thawed rapidly at 37°C and transferred into DMEM-F12+10% Fetal bovine serum (FBS) in a 15 cc centrifuge tube. The cells were then centrifuged at 800 rpm for five minutes. The supernatant was discarded and the cells resuspended in DMEM+1% FBS in 48 well plates. The post-thaw viability was assessed by trypan Blue dye exclusion method prior to seeding at a density of 1×10^5 cells/cm^2. After 24

hours, the medium was changed to serum-free DMEM:F12 supplemented with gentamicin, ITS, and retinol.

The dose response study was performed by adding thawed cryopreserved porcine Sertoli cells to the hNT neurons in the wells at the following ratios (SC:hNT): 0:1, 1:1, 2.5:1, 5:1. The control for the dose-response was no Sertoli cells.

Immunocytochemistry and quantification of cell numbers, soma size, neurite outgrowth and number of branching points: Immunocytochemistry was performed using a monoclonal primary antibody against TH (1:2000, Incstar) and as a secondary antibody, biotinylated horse anti-mouse (1:300, Vector, Burlingame, CA, USA). The antibody complex was developed using avidin-biotin complex (ABC-elite kit; Vector) (Diaminobenztropine (DAB-kit, Vector) was used to visualize the developed product.

The survival or induction of TH-positive neurons was assessed at x200 magnification in a blind coded manor using a 400 μm ocular grid to cover almost 2% of the total area when placed in 10 placements for each well. The morphological evaluation was conducted at a co-culture ratio of 1:1, and 50 cells from 5 independent cultures were evaluated by measurement with the above mentioned reticule grid at x200 magnification.

Isolation and Pretreatment of Sertoli Cells and Peritubular Cells: As previously described (Cameron and Muffly, 1991) decapsulated rat or other mammalian testes

were subjected to sequential enzymatic treatment at 37°C using 0.25% trypsin (Sigma) and 0.1% collagenase (Sigma, type V) (Cameron et al. 1987a; Cameron et al. 1987b). The resulting Sertoli cell aggregates were equally distributed in a volume of 20ml incubation medium into 75 cm^2 tissue culture flasks (Costar). The Sertoli cells after the last step of the isolation were plated at a density of $5x10^6$ cells/T-75 flasks (Corning) in DMEM:F12 (Life Technologies)+1% FBS supplemented with ITS (Collaborative research), retinol (Sigma), and gentamicin (Sigma). Plated Sertoli aggregates were incubated at 39°C in 5% CO_2-95% air for 48 hours which preferentially selects for Sertoli cells over germ cells. After this incubation cells were subjected to hypotonic treatment with sterile 0.5mM Tris-HCl buffer for one minute (Galdieri et al. 1981) to expedite the removal of contaminating germ cells. Following two washes with incubation medium, flasks were replenished with 20ml incubation medium and returned to the CO_2-injected incubator at 37°C in 5% CO_2-95% air. The resulting pre-treated Sertoli-enriched monocultures contained greater than 95% Sertoli cells. Plating density of $< 2.0 \times 10^6$ Sertoli cells/cm^2 generally did not result in a confluent monolayer of cells. For cryopreservation of the Sertoli cells after 48 hours in culture the cells were frozen in DMEM:F12+10% DMSO + 10% FBS. Alternatively the cells were washed two times to be used fresh as indicated.

Incubation Medium and Sertoli Cell Conditioned Medium: The incubation (control) medium used for Sertoli cell culture and co-culture was Dulbecco's Minimum Essential Medium:Ham's F12 Nutrient Medium (Whittaker Bioproducts) mixed 1:1 and supplemented with 3mg/ml L-glutamine (Sigma, grade III), 0.01cc/ml insulin-transferrin-selenium (ITS, Collaborative Research, Inc.), 50 ng/ml retinol (Sigma), 19μl/ml lactic acid (Sigma) and 0.01cc/ml gentamicin sulfate (Gibco).

Following the first 48 hour incubation period of isolated Sertoli cells (rat or porcine as indicated in text), conditioned media was collected and centrifuged at 1500rpm for five minutes. The supernatant was collected and immediately frozen in sterile test tubes. This medium was identified as Sertoli conditioned medium (SCM).

EXAMPLE 1

Enhanced Viability of Cryopreserved Cells and Post-Thaw Viability with Sertoli Cells and Sertoli Cell Conditioned Medium (SCM):

Cell transplantation therapies are optimized by the availability of cryopreserved cells which have high post-thaw viability. Fetal brain cells are not cryopreserved well. To enhance the post-thaw recoverability and viability of fetal brain cells (FBC), the cryoprotectant properties of Sertoli cells and

Sertoli cell pre-conditioned medium (SCM) on rat fetal brain cells (FBC) including ventral mesencephalon, commercially available immature brain cells (hNT) and striated lateral and medial eminence cells were investigated.

Fetal brain cells (FBC) were collected from the ventral mesencephalon of fetal rats (15-17 days gestation). The fetal brain tissue was suspended in medium and initially dispersed by passing it through a series of sequentially decreasing sized hypodermic needles (18-26 gauge). The resulting suspension was treated with 0.1% trypsin for five minutes and followed by 0.1% trypsin inhibitor for two minutes. The suspended FBC were washed (3x), resuspended in incubation medium and plated in poly-L-lysine-coated culture vessels. The hNT cells are derived from NT2 cells (STRATAGENE).

Studies utilizing rat Sertoli Cells:

hNT cells or cells from the ventral mesencephalon (VM) of embryonic E15 rats were isolated and either cryopreserved alone, with rat Sertoli cells (SC) (Figure 1A) or incubated in rat SCM after thawing and post-thaw viability determined. Cryopreservation with Sertoli Cells significantly increased post-thaw viability of FBS (Figure 1A) and hNT. Additionally, it was determined that incubation in SCM after thawing increased post-thaw viability.

Monocultures of FBC and hNT cells (approximately 5 x 10^6/ml) were cryopreserved at high density in SCM+10% DMSO or control medium+10% DMSO and stored in liquid nitrogen. Quickly thawed cells were washed and resuspended in warm SCM. Samples were collected for cell number and live/dead percent estimations by duplicate, blind counts by three separate individuals.

The percent of post-thaw live cells, estimated by vital dye exclusion, doubled for FBC and hNT cells cryopreserved in SCM when compared to cells cryopreserved in control medium. The mean post-thaw hNT cells recovered from SCM medium (2.0×10^6/ml) was significantly ($P<.05$) greater than the mean post-thaw hNT recovered from control medium (1.4×10^6/ml).

PERCENT INCREASE OF VIABILITY OVER CONTROLS

FBC + SCM	10%
hNT + SCM	43%

The results show that media soluble factors secreted by Sertoli cells enhanced the post-thaw viability of FBC, and hNT cells.

Studies Utilizing Porcine Sertoli Cells:

Figures 1B and 1C demonstrate that rat FBC and hNT cells, respectively, when co-cryopreserved (as described herein above) with porcine Sertoli cells have enhanced post-thaw viability.

The number of rat FBC recovered following cryopreservation (Figure 1D) was significantly increased ($p<0.05$) when the cells were cryopreserved with porcine Sertoli cells (Mean ± SEM: 2×10^6/ml ± 0.2×10^6/ml) or cryopreserved in porcine SCM (2×10^6/ml ± 0.2×10^6/ml) when compared to FBC cells cryopreserved in control medium only (1.4×10^6/ml ± 0.2×10^6/ml). The percent of post-thaw viable cells, estimated by vital dye exclusion was significantly increased ($p<0.05$) for FBC cryopreserved with Sertoli Cells when compared to FBC cryopreserved without Sertoli Cells.

Cryopreserved VM cells were thawed and incubated with or without Sertoli cells for 24 hours. The presence of Sertoli Cells in the post-thaw co-culture (SC:VM:1:1) significantly ($p<0.05$) increased the number of TH-immunopositive VM cells (592 ± 177) when compared to past-thaw VM cell monoculture (224 ± 77) plated at the same density (Figure 1E).

EXAMPLE 2

<u>The effect of rat Sertoli Cell Conditioned Media on rat VM cells</u>:

To provide evidence for secretion and release of trophic factors by Sertoli Cells, fetal dopaminergic neurons were cultured in Sertoli Cell conditioned medium (SCM). As described herein above, fetal ventral

mesencephalon cells were obtained from E15 rats and were cultured for seven days with or without rat SCM (Figures 2-3). Referring to Figure 2B, VM cells incubated in SCM were highly stimulated as compared to the control (Figure 2A). Figure 2C illustrates that at higher magnification, VM cells incubated in SCM show neurite outgrowth.

After 7 days in culture, the cells were fixed with 4% formaldehyde and stained for Tyrosine Hydroxylase (TH) immunoreactivity (Figure 3). The number of TH-positive cells showed a significant increase ($p<0.028$) over the control in these culture conditions (Figure 4).

EXAMPLE 3

The effect of Sertoli cells on rat embryonic DA neurons:

To determine the survival and trophic effect of porcine Sertoli cells on rat embryonic mesencephalic DA neurons, Applicants co-cultured fresh or thawed cryopreserved primary porcine Sertoli cells and E15 mesencephalic neurons in supplemented media on poly-L-lysin coated 48 well tissue culture plates. After 7 days in culture, the cells were fixed with 4% formaldehyde and stained for Tyrosine Hydroxylase (TH) immunoreactivity (Figure 5).

The trophic effect was assessed by measuring soma size, neuritic outgrowth, and number of branching points in a culture with Sertoli:VM ratio of 1:1 (Figure

6A-D). The TH number was estimated in the culture and showed an significant increase in TH-positive neurons with 3198+478 in the treated group compared to control 712±121 (p=0.028) (Figure 6A). The Sertoli co-cultured TH-positive cells showed a significant 2.4-fold increased soma size 31.85±2.23 μm (p<0.005) compared to control 13.23±1.01 μm, (Figure 6B). The increase in length (228±13 μm) of the longest neurite differed significantly, (p<0.0001) in the treated group (Figure 6C), when compared to the control (49±3 μm), which represents a 4.6-fold increased sprouting of the longest neurite per cell. The number of branching points was increased from 1.12±0.166 in the control to 3.74±0.183 in the Sertoli co-cultured DA-neurons (Figure 6D).

To determine the effect of freshly isolated porcine Sertoli cells and to exclude an effect generated by contaminating cells, Applicants conducted a study with fresh porcine Sertoli and Peritubular cells, consisting of five different ratios (1:1000, 1:100, 1:10, 1:1 and 5:1) (Figure 7). The 1:1 ratio of Sertoli:VM cells showed a significant 3.2-fold increase in the number of TH-positive neurons (4183±177) compared to control (1297±127). No effect was seen with peritubular cells on increase of TH-positive neurons at any of the ratios. However a significant decrease (p=0.003) in the number of TH-positive cells (132±34) was seen at the 5:1 ratio of Peritubular:VM compared to control.

To determine if the survival and trophic effect of cryopreserved porcine Sertoli cells would generate the same results as freshly isolated Sertoli Applicants performed a dose-response study with six different ratios of cryopreserved Sertoli cells and E15 VM neurons (1:100, 1:10, 1:2.5, 1:1, 2.5:1, and 5:1) (Figure 8). Maximal responses were seen at SC:VM ratios of 1:5, 1:2.5 and 1:1, with the ratio 1:5 showing 3570 ± 139 TH-positive cells, and the 1:1 ratio resulting in 3437±740, which is a 5.6-fold significant increase (p=0.0012), compared to control with 612±72 cells. The ratio 5:1 had a significantly decreased number of TH-positive cells, 224±102, compared to the ratios 1:5-1:1 (p=0.0014).

EXAMPLE 4

<u>The effect of Sertoli cells on VM and hNT cells in culture</u>:

To investigate whether porcine Sertoli cells had an effect on the induction of Tyrosine Hydroxylase (TH) of cryopreserved hNT neurons and/or induction of phenotypic appearance, Applicants conducted a dose-response study with three different concentrations; 1:1, 2.5:1, and 5:1 (Sertoli cells:hNT). The results showed an increase in number of TH-positive neurons and a morphological change in maturation. The number of TH-positive cells, was significantly increased for all ratios. The most pronounced effect, a nearly sixfold

increase ($p<0.0001$), was found in the co-cultures with a ratio of 5:1 Sertoli cells:hNT neurons, in which 7262±1348 TH-positive neurons survived for 7 days *in vitro* compared to 1215±203 in the control (Figure 9). The mean post-thaw viability was 65% of the hNT neurons.

In the control cultures, the cells tended to stay in clusters (Figure 10A), but when co-cultured with Sertoli cells the hNT cells were migrating and also extended longer neuritic processes (Figure 10B) as assessed morphologically in a phase contrast microscope.

EXAMPLE 5

<u>The effect of porcine Sertoli Cell Conditioned Media on rat VM cells</u>:

Tissue for cell culture was obtained from embryonic day 15 rat (Sprague Dawley, Civic Miller) embryos CRL 13 mm as described herein above. Briefly, ventral mesencephalon (VM) cell suspensions were prepared as previously described by Othberg et al., [1995]. Cell concentration was assessed using the trypan blue dye exclusion method and the cells were frozen at a concentration of 5×10^6 cells/ml. The freezing was performed using 10% fetal bovine serum (FBS)+10%DMSO in DMEM:F12 for the control and in porcine Sertoli pre conditioned media (10% FBS+10%DMSO in DMEM:F12) in the treated group.

The cells were then frozen at -70C at a rate of one degree per minute. Following the cryopreservation and freezing the cells were thawed at 37°C and transferred to DMEM:F12+10%FBS. The cells were then centrifuged at 700 rpm for 3 minutes, and the cells were re-suspended in 1 ml serum-free DMEM:F12 and the cell number and viability was assessed with trypan blue dye exclusion method.

Thereafter, the cells were plated in poly-L-lysine (Sigma) coated 8-well chamber slides (Nunc) in DMEM:F12 for the control group or porcine Sertoli preconditioned serum-free media for the treated group at a density of 100,000 cells/cm^2. They were thereafter fixed after 24 hours and tyrosine hydroxylase (TH) immunohistochemistry was performed as described herein above. The cell number was assessed by counting all TH-immuno reactive cells per wells. Statistical analysis was performed by using an unpaired t-test.

<u>Results</u>

Control cultures showed a survival of 59.75 ± 5 cells/well. VM cells that were frozen and thawed out in porcine Sertoli cell conditioned media showed a significant increase in survival of 104 ± 21.6 cells/well ($p=0.0672$). The porcine Sertoli cell preconditioned media provided a 2-fold increase in surviving TH-positive cells.

EXAMPLE 6

SURVIVAL OF SERTOLI CELLS IN THE BRAIN

Sertoli cell/chromaffin cell co-grafts were transplanted into the striatum of the brain (Sanberg et al, 1996). Transplanted chromaffin cells were present and easily identified because of the inclusion of secretory granules unique to the cells. Co-transplanted Sertoli cells were detected immediately adjacent to the electron dense chromaffin cells. This demonstrates the survival of co-grafted adrenal chromaffin cells with Sertoli cells in the brain.

EXAMPLE 7

Effects of Cyclosporine A (CsA) on the survival of transplanted Sertoli cells:

Fluorescent cell labeling: Immediately prior to transplantation (approximately two hours), Sertoli cell monocultures prepared as described herein were treated with CM-DiI fluorescent dye for cell tracking (100(1 stock/ml medium; Molecular Probes, Inc., Eugene, OR) for seven minutes at 37°C and then placed in the refrigerator (4°C) for an additional 15 minutes. Fluorescent "tagged" Sertoli cells were washed (three times) and resuspended in 1ml of incubation medium.

The effect of cyclosporine A on the survival of grafted Sertoli cells in situ was examined. Grafted Sertoli cells were labeled with a fluorescent tag (DiI)

prior to transplantation into the striatum of the brain. The tissue was collected one month post-transplantation. Viable fluorescent Sertoli cells were seen in a rat host that had not received immunosuppression therapy with cyclosporine A. Viable fluorescent Sertoli cells are found in a rat host that did receive cyclosporine A immunosuppression therapy. This example demonstrates that cyclosporine A is not necessary for the survival of Sertoli cells transplanted into the brain.

Throughout this application, various publications, including United States patents, are referenced by citation or number. Full citations for the publications are listed below. The disclosures of these publications and patents in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

The invention has been described in an illustrative manner, and it is to be understood that the terminology which has been used is intended to be in the nature of words of description rather than of limitation.

Obviously, many modifications and variations of the present invention are possible in light of the above teachings. It is, therefore, to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described.

TABLE 1

Category and Protein	**Function**
Hormones/Growth Factors	
Mullerian Inhibiting Substance	inhibits Mullerian duct
inhibin	inhibits FSH release
Insulin-like Growth Factor (Sommatomedins A and C, IGF)	growth factor
Prodynorphin	
Interleukin-1α	mitogen
Transforming Growth Factor α & β	growth factors
Basic Fibroblast Growth Factor	growth factor
LHRH-like Factor	Leydig cell steroidogenesis
Sertoli Secreted Growth Factor	growth factor
Seminiferous Growth Factor	
Leydig Cell Stimulatory Activity	
Testins	
CMB proteins	
Vitamin Binding Proteins	vitamin transport
Transport and Bioprotection	
Transferrin	iron transport
Ceruloplasm	copper transport
Saposin SGP-1	binds glycosphinogolipids
SGP-2 (Clusterin)	lipid transport?
Androgen Binding Protein	transports T and DHT
SPARC	calcium binding protein?
IGF Binding Proteins	IGF transport
Riboflavin Binding Protein	riboflavin transport
Proteases and Protease Inhibitors	
Plasminogen Activator	protease
Cyclic Protein-2	protease inhibitor
Cystatin	protease inhibitor
α_2-Macroglobulin	protease inhibitor
Type IV Collagenase	protease
Metalloproteinases	protease
Basement membrane	
Collagen IV	
Laminin	
Proteoglycans	
Immunoprotective Factors	
Fas-L	
Activin	

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CLAIMS

What is claimed is:

1. A method to increase survival and maturation of cells for transplantation including the step of culturing the cells with sertoli-cell conditioned media.

2. The method as set forth in claim 1 wherein said culturing step is further defined as the cells are dopaminergic neuronal cells.

3. The method as set forth in claim 2 wherein said culturing step is further defined as the dopaminergic neuronal cells are of fetal origin.

4. The method as set forth in claim 2 wherein said culturing step is further defined as the dopaminergic neural cells are derived from a human teratocarcinoma cell line.

5. A method of producing sertoli-cell conditioned media by the step of culturing sertoli cells in a culture media.

6. The method as set forth in claim 5 further including the step of removing the conditioned medium from the Sertoli cells.

7. The method as set forth in claim 5, wherein said culturing step is further defined as culturing the Sertoli cells for a period of time ranging from approximately one hour to approximately seven days.

8. The method as set forth in claim 7, wherein the time period is 48 hours.

9. The method as set forth in claim 5, wherein the medium includes retinol, lactic acid and insulin-transferrin-selenium.

10. The method as set forth in claim 5, wherein the culturing step is further defined as the concentration of the Sertoli cells ranges from approximately 1×10^4 cells/cm^2 to approximately 1×10^7 cells/cm^2.

11. The method as set forth in claim 10, wherein the concentration is 6×10^5 cells/cm^2.

12. The method as set forth in claim 5, wherein the culture temperature is 39°C

13. The method as set forth in claim 5 wherein the culture medium is selected from the group consisting of DMEM:Ham's F12, X vivo-10 and X vivo-15.

14. A sertoli cell conditioned media produced by culturing 1×10^4 to approximately 1×10^7 sertoli cells/cm^2 in a culture medium for approximately one hour to approximately seven days.

15. The conditioned media as set forth in claim 14 wherein the culture medium is selected from DMEM:Ham's F12, X vivo-10 and X vivo-15.

16. A method of enhancing the viability of cryopreserved cells, said method comprising the steps of:

adding sertoli cell conditioned medium as set forth in claim 14 to cells to be cryopreserved.

17. The method as set forth in claim 16, wherein the cells to be cryopreserved include cells of the central nervous system, lymphocytes, hybridomas, fibroblasts, cells for gene therapy, fetal cells, myoblasts, endocrine cells, hepatocytes, endothelial cells and the like.

18. A method for enhancing the viability of cryopreserved cells, said method comprising the steps of:

co-culturing Sertoli cells and cells to be cryopreserved in media and cryopreserving together both the Sertoli cells and cells to be cryopreserved.

19. A method as set forth in claim 18 wherein said culturing step is further defined as co-culturing the Sertoli cells and cells to be cryopreserved for a period of time ranging from approximately one hour to approximately seven days.

20. A method of enhancing the viability of cryopreserved cells to be thawed, said method comprising the steps of:

adding sertoli cell conditioned medium as set forth in claim 14 to cryopreserved cells upon thawing.

21. A method of improving survival of a graft *in situ* by treating the graft *in situ* with sertoli-cell conditioned media as set forth in claim 14.

22. A method of improving wound healing *in situ* by treating the wound *in situ* with sertoli-cell conditioned media as set forth in claim 14.

23. The method as set forth in claim 22 wherein said treating step is further defined as the wound is an injured spinal cord.

24. A method to increase survival and maturation of cells for transplantation including the step of culturing the cells with Sertoli cells.

25. The method as set forth in claim 24 wherein said culturing step is further defined as the cells are dopaminergic neuronal cells.

26. The method as set forth in claim 25 wherein said culturing step is further defined as the dopaminergic neuronal cells are of fetal origin.

27. The method as set forth in claim 25 wherein said culturing step is further defined as the dopaminergic neural cells are derived from a human teratocarcinoma cell line.

28. A method to induce phenotypic change in cells for transplantation by culturing the cells to be transplanted with an agent selected from the group consisting of Sertoli cells and sertoli cell condition media.

29. The method as set forth in claim 28 wherein the cells to be transplanted are islets cells, myoblasts, human or animal neurons including striatal neurons and septal neurons, cells from a human teratocarcinoma neuronal cell line, chromaffin cells, hepatocytes, ventral mesencephalic cells, skin grafts, and bone marrow.

30. The method as set forth in claim 29 wherein the cells from a human teratocarcinoma neuronal cell line cultured with Sertoli cells or Sertoli cell condition medium are tyrosine-hydroxylase positive.

31. A composition, containing a sertoli-cell conditioned media as set forth in claim 14, as an active ingredient for use in improving survival of a graft *in situ*.

32. A composition, containing a sertoli-cell conditioned media as set forth in claim 14, as an active ingredient for use in improving wound healing *in situ*.

33. The composition as set forth in claim 32 wherein said wound is an injured spinal cord.

34. A composition, containing Sertoli cells as an active ingredient, to increase survival and maturation of cells for transplantation.

35. The composition as set forth in claim 34 wherein said cells are dopaminergic neuronal cells.

36. The composition as set forth in claim 35 wherein said dopaminergic neuronal cells are of fetal origin.

37. The composition as set forth in claim 35 wherein said dopaminergic neural cells are derived from a human teratocarcinoma cell line.

38. A composition, containing a sertoli-cell conditioned media as set forth in claim 14, as an active ingredient for use in enhancing the viability of cryopreserved cells.

39. A composition, containing Sertoli cells, as an active ingredient for enhancing the viability of cryopreserved cells, by co-culturing Sertoli cells and cells to be cryopreserved in media and cryopreserving

together both the Sertoli cells and cells to be cryopreserved.

40. A composition, containing sertoli cell conditioned media as set forth in claim 14, as an active ingredient for enhancing the viability of cryopreserved cells upon thawing.

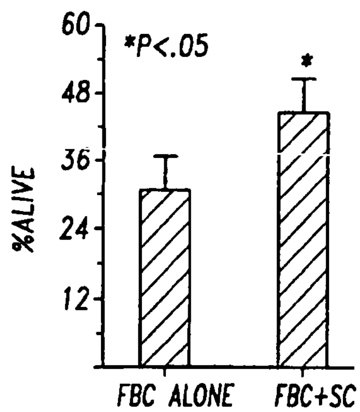

Fig-1A

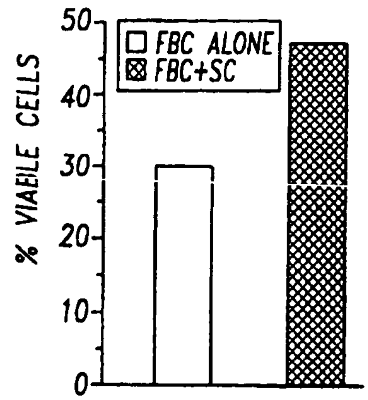

Fig-1B

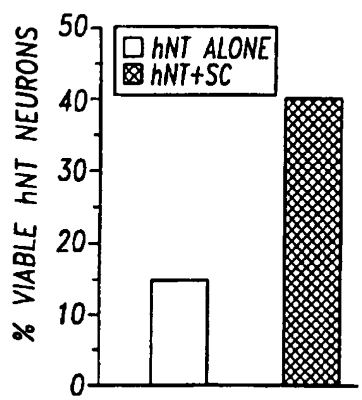

Fig-1C

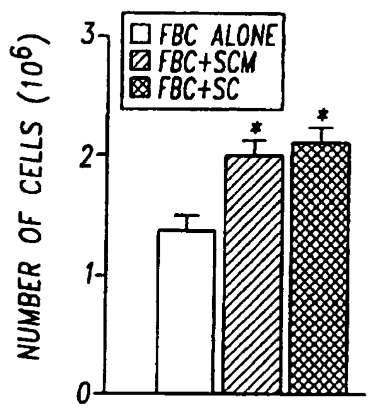

Fig-1D

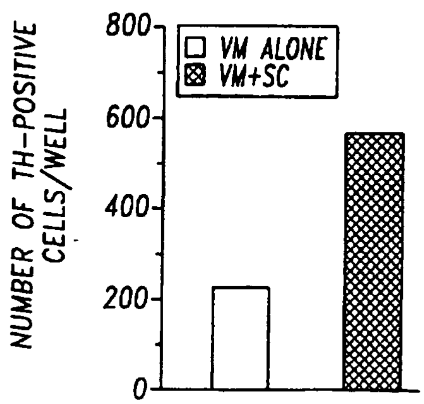

Fig-1E

Fig - 2A

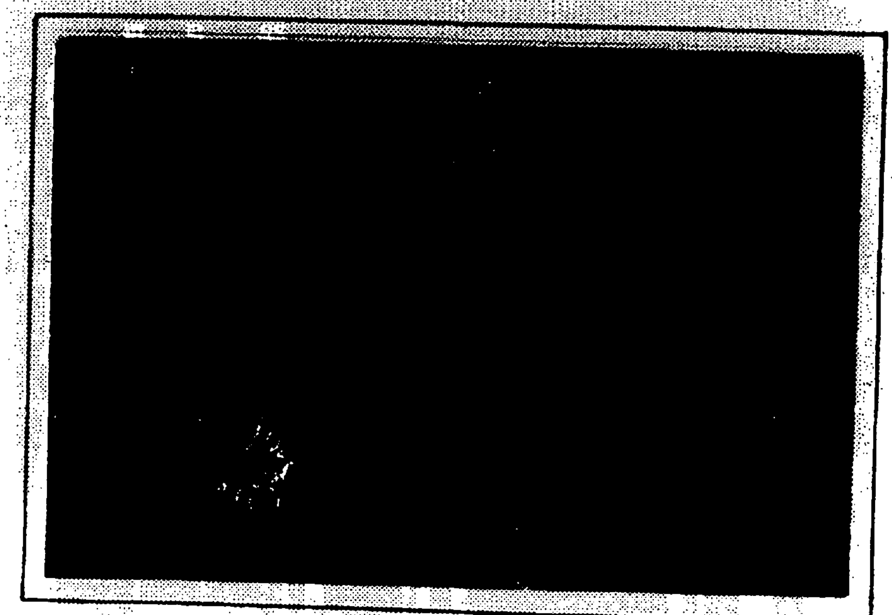

Fig - 2B

Fig - 2C

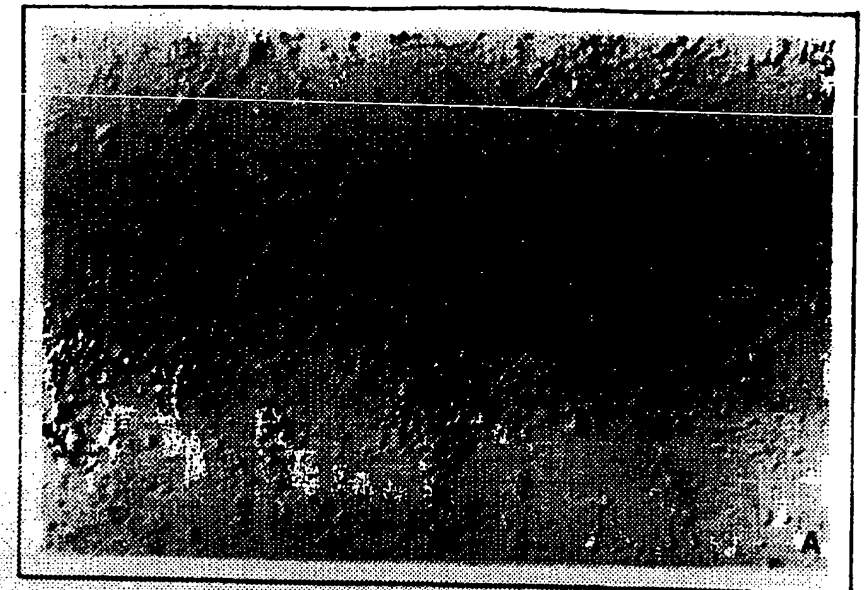

Fig - 3A

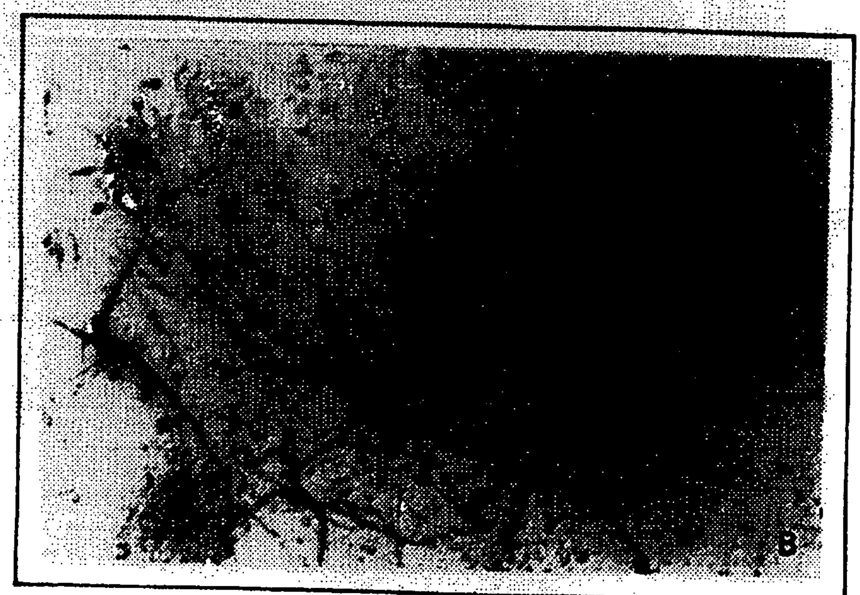

Fig - 3B

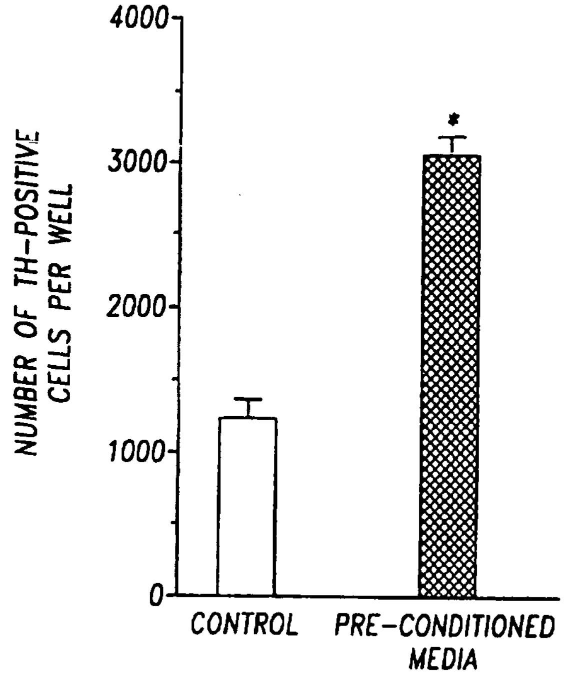
4000
3000
2000
1000
0
NUMBER OF TH-POSITIVE CELLS PER WELL
*
CONTROL
PRE-CONDITIONED MEDIA

Fig-4

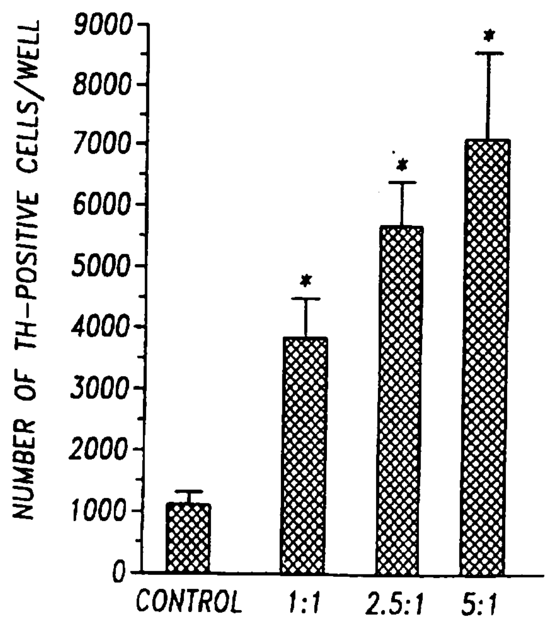
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
NUMBER OF TH-POSITIVE CELLS/WELL
*
*
*
CONTROL
1:1
2.5:1
5:1

Fig-9

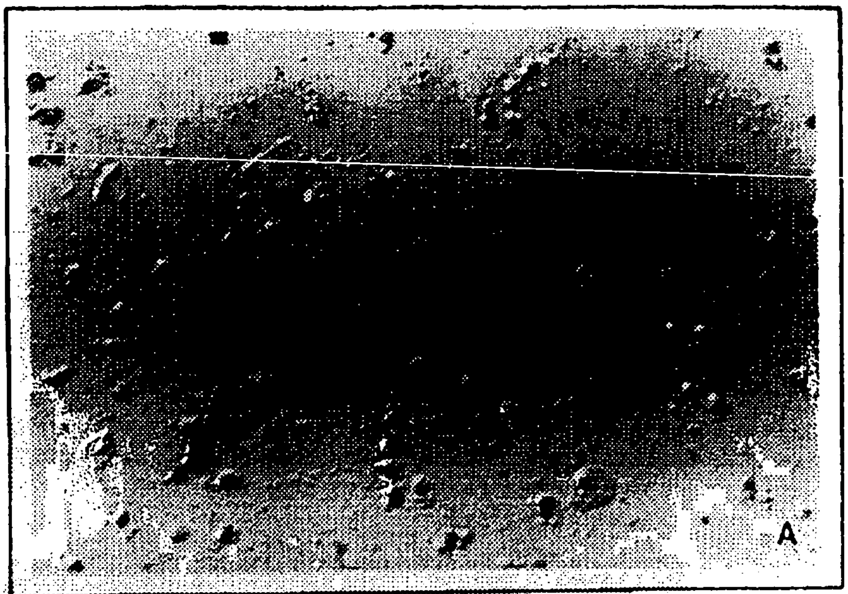

Fig - 5A

Fig - 5B

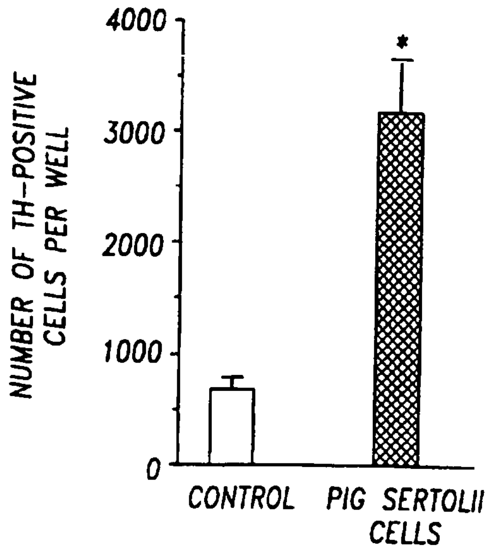

Fig-6A

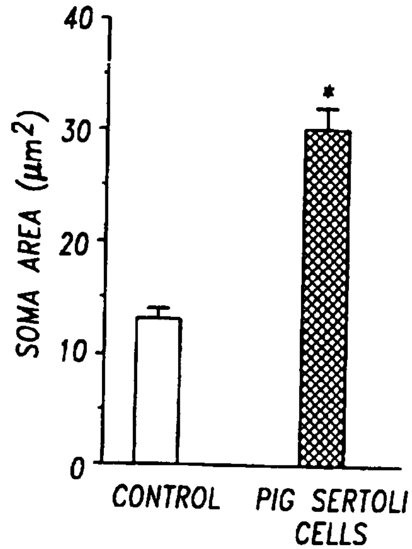

Fig-6B

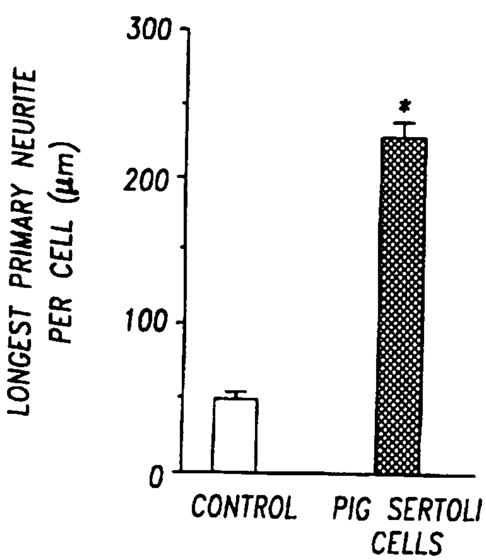

Fig-6C

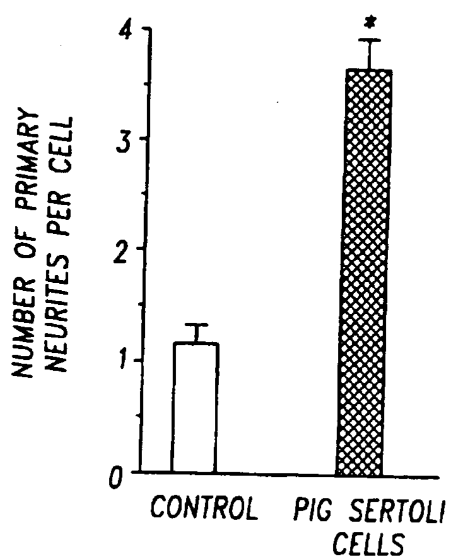

Fig-6D

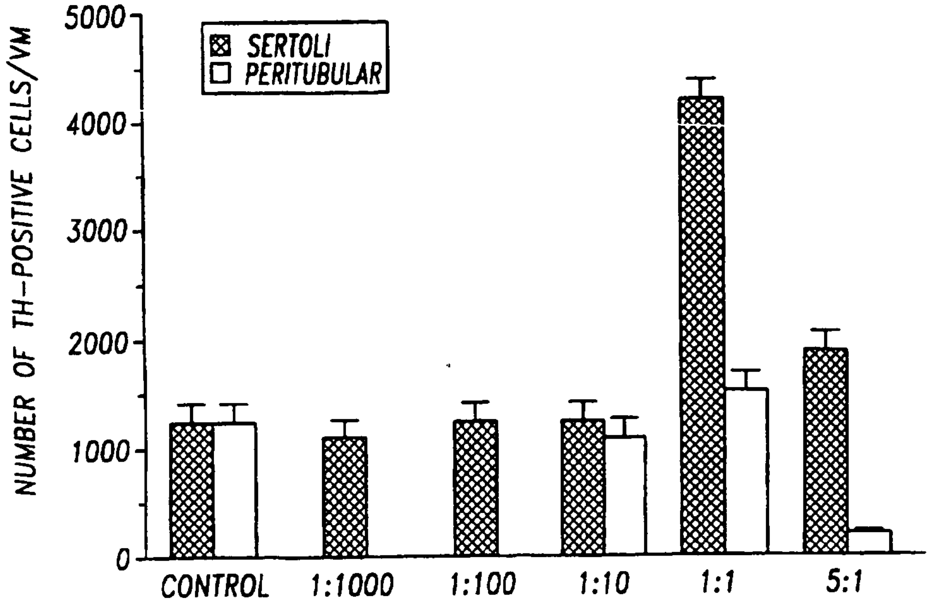
NUMBER OF TH-POSITIVE CELLS/VM
5000
4000
3000
2000
1000
0
SERTOLI
PERITUBULAR
CONTROL
1:1000
1:100
1:10
1:1
5:1

Fig-7

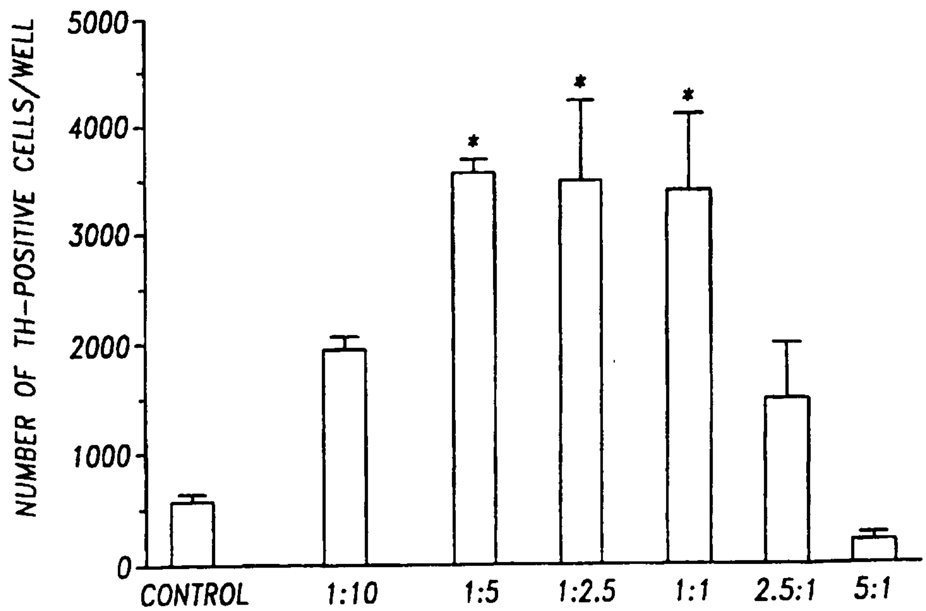
NUMBER OF TH-POSITIVE CELLS/WELL
5000
4000
3000
2000
1000
0
*
CONTROL
1:10
1:5
1:2.5
1:1
2.5:1
5:1

Fig-8

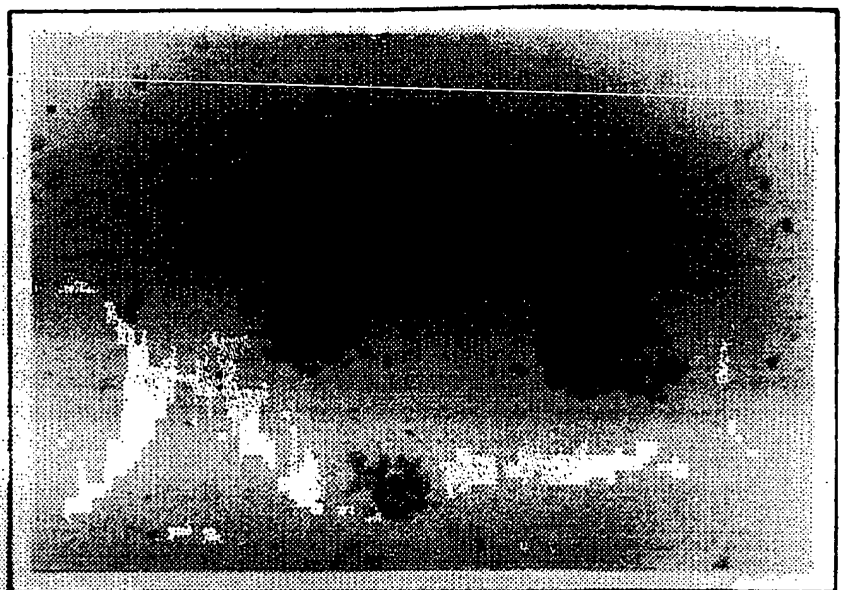

Fig - 10A

Fig - 10B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/03911

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A01N 1/00; C12N 5/00
US CL :424/93.3, 93.7; 435/374, 377, 404
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93.3, 93.7; 435/374, 377, 404

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG, MEDLINE, EMBASE, BIOSIS
search terms: sertoli, cryopreservation, media, medium

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SELAWRY, H.P. et al. Production of a Factor, or Factors, Suppressing IL-2 Production and T Cell Proliferation by Sertoli Cell-enriched Preparations. Transplantation. November 1991, Vol. 52, No. 5, pages 846-850, especially page 847.	1-40

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
28 MAY 1997

Date of mailing of the international search report
11 JUL 1997

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
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Washington, D.C. 20231
Facsimile No. (703) 305-3230

Authorized officer
EMMA CECH
Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/03911

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claims 1-4, 14, 15, 21, 24-33, 38, and 40, drawn sertoli condition media and to a method to increase survival of cells for transplantation.

Group II, claims 5-13, drawn to a method to produce sertoli cell conditioned media.

Group III, claims 16-20, drawn to a method for enhancing the viability of cryopreserved cells.

Group IV, claims 22 and 23, drawn to a method of improving wound healing.

Group V, claim(s) 34-37 and 39, drawn to a composition comprising sertoli cells.

The inventions listed as Groups I, II, III, IV, and V do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Groups II, III, IV, and V do not contain the special technical feature of cell transplantation as Group I. Accordingly the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.